

MrkD_{1P} from *Klebsiella pneumoniae* Strain IA565 Allows for Coexistence with *Pseudomonas aeruginosa* and Protection from Protease-Mediated Biofilm Detachment

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Biofilm formation and persistence are essential components for the continued survival of pathogens inside the host and constitute a major contributor to the development of chronic wounds with resistance to antimicrobial compounds. Understanding these processes is crucial for control of biofilm-mediated disease. Though chronic wound infections are often polymicrobial in nature, much of the research on chronic wound-related microbes has focused on single-species models. *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* are microbes that are often found together in wound isolates and are able to form stable *in vitro* biofilms, despite the antagonistic nature of *P. aeruginosa* with other organisms. Mutants of the *K. pneumoniae* strain IA565 lacking the plasmid-borne *mrkD*_{1P} gene were less competitive than the wild type in an *in vitro* dual-species biofilm model with *P. aeruginosa* (PAO1). PAO1 spent medium inhibited the formation of biofilm of *mrkD*_{1P}-deficient mutants and disrupted preestablished biofilms, with no effect on IA565 and no effect on the growth of the wild type or mutants. A screen using a two-allele PAO1 transposon library identified the LasB elastase as the secreted effector involved in biofilm disruption, and a purified version of the protein produced results similar to those with PAO1 spent medium. Various other proteases had a similar effect, suggesting that the disruption of the *mrkD*_{1P} gene causes sensitivity to general proteolytic effects and indicating a role for MrkD_{1P} in protection against host antibiofilm effectors. Our results suggest that MrkD_{1P} allows for competition of *K. pneumoniae* with *P. aeruginosa* in a mixed-species biofilm and provides defense against microbial and host-derived proteases.

Despite medical advances that have decreased mortality from infectious agents, recurrent and chronic wound infections are still a growing burden on the global population (1). Microorganisms that are often associated with these chronic wound infections exist in persistent sessile communities known as biofilms that consist of adherent cells contained within an extracellular polymeric substance (EPS) composed of polysaccharides, DNA, and proteins (2, 3). These communities are phenotypically distinct from their planktonic counterparts, providing the organisms with protection from various antimicrobial agents (4, 5). Biofilm development is associated with over half of all human chronic infections and is essential to the continued survival of pathogens where the host utilizes many biofilm countermeasures, including phagocytosis and secretion of effectors such as proteases, lactoferrin, oxygen radicals, and defensins, among others (6–9). Naturally occurring, biofilm-mediated chronic wound infections are typically polymicrobial in nature and can be composed of numerous species of bacteria and fungi (10). Mutualistic, commensal, and competitive relationships have been observed between various sets of organisms in mixed communities, adding to the already complex nature of the biofilm (11–13). New virulence factors that attack both the host and competing species have also been identified from these organisms (14, 15). Still, much of the research on chronic wound-related microbes has, to this point, focused on single-species models, making it likely that important virulence factors, targets, and disease mechanisms are being missed.

Klebsiella pneumoniae and *Pseudomonas aeruginosa* are two biofilm-producing organisms that are often found together in chronic wound infections (10). *K. pneumoniae* is a Gram-negative bacterium that is found ubiquitously in both terrestrial and aquatic environments (16). The organism is responsible for respi-

ratory illnesses and urinary tract infections (UTIs) but is an increasing cause of infection at surgical sites and of chronic wound infections (17). The emergence of extended-spectrum β -lactamase (ESBL)- and carbapenemase-producing strains is a cause of growing concern in the medical community (18). Biofilm formation in *K. pneumoniae* is a major virulence factor used to colonize the human host (19). This activity is mediated by a type 3 fimbria that is used for adherence to various forms of extracellular matrix (ECM) proteins, such as collagen or fibronectin (19, 20). The recognition of each type of ECM protein is mediated by various forms of the fimbrial tip protein MrkD (21). For instance the MrkD_{1P} form, found in very few strains of *K. pneumoniae* (but found on a stable plasmid in strain IA565, a strain whose biofilm formation is well studied), binds to type V collagen, while MrkD_{1C}, found on the chromosome in a vast majority of strains, including IA565, binds to collagen types IV and V (22). Although not essential for biofilm production, MrkD plays a role in the physiology of the *K. pneumoniae* biofilm and is an important factor in pathogenesis (19, 20).

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TABLE 1 Strains and plasmids used in this study

Strain or plasmid	Description	Source or reference
<i>P. aeruginosa</i>		
PAO1	Wild-type reference strain	43
PA0401	Mutant PW1741, noncatalytic dihydroorotase-like protein	37
PA0697	Mutant PW2265, hypothetical protein	37
PA3135	Mutant PW7392, probable transcriptional regulator	37
PA2478	Mutant PW5161, probable thiol:disulfide interchange protein	37
PA3742	Mutant PW7303, elastase LasB	37
PA4280	Mutant PW8208, BirA bifunctional protein	37
PA0502	Mutant PW1924, probable biotin biosynthesis protein BirH	37
PA1684	Mutant PW3991, 1,2-dihydroxy-3-keto-5-methylthiopentene dioxygenase MtnD	37
PA3383	Mutant PW10408, binding protein component of ABC phosphonate transporter	37
<i>K. pneumoniae</i>		
IA565	Wild type containing chromosome- and plasmid-borne <i>mrk</i> gene cluster	44
IApc35	Fimbriate and nonhemagglutinating strain of IA565, lacking plasmid-borne <i>mrk</i> gene cluster	44
BC2	IA565 with <i>Himar1</i> transposon insertion in the plasmid-borne <i>mrkD</i> _{1P} gene	This study
UIR082	Strain possessing <i>mrkD</i> _{1C} but not <i>mrkD</i> _{1P}	36
UIR207	Strain possessing <i>mrkD</i> _{1C} but not <i>mrkD</i> _{1P}	36
UI917	Strain possessing <i>mrkD</i> _{1P} but not <i>mrkD</i> _{1C}	36
UI925	Strain possessing <i>mrkD</i> _{1P} but not <i>mrkD</i> _{1C}	36
THK12113	Strain possessing <i>mrkD</i> _{1C} but not <i>mrkD</i> _{1P}	36
CAS55	C3091 <i>K. pneumoniae</i> UTI clinical isolate	45
<i>E. coli</i> NEB10 α		New England Biolabs
Cloning vectors		
pGEM-T Easy		Promega
pACYC184		New England Biolabs
Recombinant plasmids		
pFK52	Plasmid-borne <i>mrkD</i> gene from <i>K. pneumoniae</i> IA565 cloned into pACYC184	46
pBMC12	pACYC plasmid with kanamycin resistance marker in place of chloramphenicol resistance marker	This study
pBMC13	pFK52 plasmid with kanamycin resistance marker in place of chloramphenicol resistance marker	This study
pBMC14	Chromosomal-borne <i>mrk</i> gene cluster from <i>K. pneumoniae</i> IA565 cloned into pGEM-T Easy	This study
pMrkD	<i>mrkD</i> gene from C3091 cloned into low-copy-no. vector pGB17	45
pKEK1410	Suicide vector pFD1 carrying a chloramphenicol marker and encoding <i>Himar1</i> -derived transposon and <i>Himar1</i> transposase	Gift of Karl E. Klose

P. aeruginosa is a ubiquitous organism that is able to form biofilms on numerous biotic and abiotic surfaces and causes a wide range of infections, from lung infections with cystic fibrosis patients to urinary tract and kidney infections, which are often attributed to contaminated medical devices (23, 24). *P. aeruginosa*, though often found with other organisms in wound infections, maintains an antagonistic relationship with many other wound-related organisms *in vitro*, including *Staphylococcus aureus*, *Staphylococcus epidermidis*, and *Burkholderia cepacia* (15, 25, 26). This outcome is due to the large number of secreted effectors, such as phenazines, rhamnolipids, *cis*-2-decenoic acid, alkaline protease, exotoxins, and elastase, which are used by *P. aeruginosa* to outcompete other organisms (27–31). Interestingly, it has previously been demonstrated that *K. pneumoniae* forms stable dual-species biofilms with *P. aeruginosa*, suggesting the presence of defense mechanisms capable of withstanding the onslaught of these *P. aeruginosa* effectors (32).

Though the structure of a dual-species *K. pneumoniae*/*P. aeruginosa* biofilm has previously been studied, the interactions between the two organisms have yet to be fully understood. The aim of this study was to identify the protective properties of *K. pneumoniae* that allow for coexistence with *P. aeruginosa* in a bio-

film setting. Using the reference laboratory *P. aeruginosa* strain PAO1 and the *K. pneumoniae* strain IA565, which carries both the 1P and 1C forms of the *mrkD* gene, we identified a protective advantage conferred by the MrkD_{1P} protein in the dual-species biofilm setting to the LasB secreted protease of *P. aeruginosa*.

MATERIALS AND METHODS

Bacterial strains, plasmid construction, and culture conditions. The *K. pneumoniae* and *P. aeruginosa* strains and recombinant plasmids are listed in Table 1. PAO1 transposon mutants were obtained from the two-allele mutant library from the Washington Genome Center. *Escherichia coli* strain DH5 α was used for cloning, and WM3046 (a gift of William Metcalf, University of Illinois) was used for conjugation. Plasmids pBMC12 and pBMC13 were created by amplifying the kanamycin resistance marker from pKD4, using the primers FRT Up ScaI (5'-GCGCGCAGTA CTGTGTAGGCTGGAGCTGCTTC-3') and FRT Dn ScaI (5'-GCGCGC AGTACTCATATGAATATCCTCTTA-3'), and cloning the fragment into the ScaI site found in the chloramphenicol marker in pACYC184 and pFK52, respectively. All strains were cultured in YEPD broth (10% yeast extract, 20% peptone, 20% dextrose) or on LB plates. Antibiotics were used at the following concentrations: 20 $\mu\text{g ml}^{-1}$ or 150 $\mu\text{g ml}^{-1}$ chloramphenicol, 15 $\mu\text{g ml}^{-1}$ tetracycline, 50 $\mu\text{g ml}^{-1}$ kanamycin, and 3 mg ml^{-1} carbenicillin.

Transposon mutagenesis of IA565. The chloramphenicol-resistant derivative of pFD1 (33), pKEK1410, which encodes a *Himar1*-derived transposon and *Himar1* transposase, was introduced into IA565 by conjugation on LB medium containing 100 μ M diaminopimelic acid (DAPA) and 1 mM β -D-1-thiogalactopyranoside (IPTG). Mutants were selected on LB plates containing no DAPA with chloramphenicol. Gene disruptions in mutants defective for competition with PAO1 were identified through inverse PCR. Briefly, the chromosomal DNA of the IA565 mutant was digested with BamHI, ligated, transformed into WM3046, and plated on LB containing DAPA and chloramphenicol. Plasmids were miniprepred from the transformants and the insertion site identified by sequencing using the primer pFD1 Up (5'-ATGCATTTAATACTAGCGACGCC-3').

CI assays. A biofilm competitive index (CI) was generated using a mixture of *K. pneumoniae* and *P. aeruginosa*. The *lacZ*⁺-containing *K. pneumoniae* strains IA565, IApc35, and BC2 were each mixed with the *lacZ*-deficient *P. aeruginosa* strain PAO1 at a 1:1 ratio in a 96-well plate and allowed to form a biofilm for 24 h in YEPD medium at 37°C with shaking. The resultant biofilms were washed 3 times, resuspended in phosphate-buffered saline (PBS) by vigorous vortexing, and dilutions plated on LB containing X-Gal (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside) and IPTG. Blue (*K. pneumoniae*) and white (*P. aeruginosa*) colonies were counted, and the competitive index was calculated as the *K. pneumoniae*/*P. aeruginosa* output ratio divided by the input inoculum ratio. Significant differences in CI were calculated using one-way analysis of variance (ANOVA).

Bacterial supernatant preparation. IA565 and PAO1 were grown in YEPD medium for 16 h at 37°C, cells harvested by centrifugation at 4,000 rpm for 30 min, and the resultant supernatant sterilized by filtration. The supernatants were immediately lyophilized, and a 10 \times stock was created by reconstitution in YEPD medium. For experiments using reconstituted supernatant, a similarly lyophilized and reconstituted YEPD was used. PAO1 transposon mutants were streaked onto LB agar and a single colony inoculated into YEPD. The mutants were grown at 37°C overnight with shaking and harvested by centrifugation, and the resultant supernatant was sterilized by filtration and used immediately. Supernatants were made fresh for each experiment.

Biofilm dispersion assays. Static biofilm formation assays were performed in 96-well microtiter plates coated with human type I collagen (354269; BD Biosciences). *K. pneumoniae* strains, or *E. coli* DH5 α carrying *mrkD*_{1P} and *mrkD*_{1C} vectors, were inoculated into 200 μ l of YEPD medium at an initial optical density at 600 nm (OD₆₀₀) of 0.05 and grown at 37°C for 16 h with shaking. Supernatants were pulled from the resultant biofilms and replaced with 200 μ l reconstituted supernatants (1 \times concentration), filter sterilized PAO1 transposon mutant supernatant, or the following compounds dissolved in YEPD: 300 μ g ml⁻¹ purified elastase B (PE961; Elastin Products Company), 50 μ g ml⁻¹ porcine pancreatic elastase (E7885; Sigma), 50 μ g ml⁻¹ PMN elastase (BML-SE284; Enzo Life Sciences), 50 μ g ml⁻¹ porcine pancreatic trypsin (T0303; Sigma), 1 μ g ml⁻¹ bovine chymotrypsin (CHY5S; Sigma); 3 μ g ml⁻¹ human antichymotrypsin (A9285; Sigma), 100 μ g ml⁻¹ proteinase K (19131; Qiagen), 0.1% sodium dodecyl sulfate (SDS) (L6026; Sigma); 100 μ g ml⁻¹ polymyxin B (92283; Sigma), or 200 Kunitz units ml⁻¹ DNase (79254; Qiagen). Treated biofilms were incubated for another 16 h with shaking at 37°C. Biofilms were washed 3 times with PBS solution, stained with a 0.4% solution of crystal violet, washed 3 times with PBS again, and dissolved in 33% acetic acid, and a spectrophotometer reading was taken at OD₅₇₀.

Bacterial growth experiments. Cell growth experiments on *K. pneumoniae* strains were performed in 96-well microtiter plates. Strains were inoculated into 200 μ l of reconstituted YEPD or PAO1 supernatant at an OD₆₀₀ reading of 0.05, and readings were taken every 2 h for 16 h. To evaluate the ability of the bacteria to form a biofilm in each medium, the growth medium was removed, the wells stained with crystal violet, the biofilms dissolved with 33% acetic acid, and an OD₅₇₀ reading taken.

Visualization of cell morphology. To determine the cell morphology of *K. pneumoniae* strains, biofilm-coated MBEC pegs (Innovotech) were fixed in 2.5% glutaraldehyde in 0.1 M PBS (pH 7.2), washed 3 times in

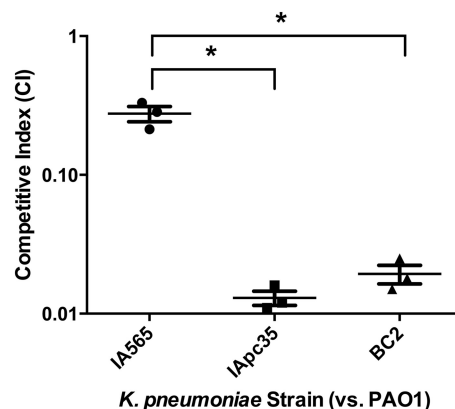


FIG 1 Mutants of *K. pneumoniae* IA565 lacking the plasmid-borne *mrkD* gene are unable to compete with *P. aeruginosa* PAO1, compared to the wild type, in an *in vitro* dual-species biofilm. Strains IA565, IApc35, and BC2 were each coinoculated with PAO1 in YEPD and allowed to form a biofilm for 24 h. Biofilms were washed, resuspended, and plated and the CFU of each strain determined. The competitive index (CI) was determined as output *K. pneumoniae*/*P. aeruginosa* divided by input. Data represent the mean \pm standard deviation of the CI from two independent experiments. Significance was determined by one-way ANOVA (*, $P < 0.01$).

PBS, and dehydrated through an ethanol series and hexamethyldisilazane. Samples were mounted with double-sided tape to specimen stubs, followed by gold-platinum (50:50) ion coating (108 Auto Sputter Coater; Ted Pella, Inc., Redding, CA). Samples were visualized using a Carl Zeiss EVO-40 scanning electron microscope (Oberkochen, Germany) operated at the scanning voltage of 10 kV. Cell length was measured using ImageJ 1.46r software (National Institutes of Health).

RESULTS

***K. pneumoniae* strains lacking the plasmid-borne type 3 fimbrial adhesin MrkD_{1P} are at a competitive disadvantage in a dual-species biofilm with *P. aeruginosa*.** Though a dual-species *K. pneumoniae*/*P. aeruginosa* biofilm has previously been studied (32), the interactions between these two species have yet to be fully analyzed. To do this, we chose to look at the interaction of *P. aeruginosa* strain PAO1 with *K. pneumoniae* strain IA565, a strain whose biofilm development has been well studied (21, 32, 34, 35). To form a biofilm, IA565 utilizes type 3 fimbriae, coded for by two *mrk* gene clusters, one found on the chromosome and the other found on a stable plasmid (36). Each gene cluster has a markedly different *mrkD* gene; *mrkD*_{1C} is found on the chromosome, while *mrkD*_{1P} is found on the plasmid, with a 60% peptide sequence homology between the two (36). A mutant of IA565, IApc35, that has lost the plasmid-borne *mrk* gene cluster and carries a termination mutation in the *mrkD*_{1C} gene, is capable of forming biofilm that surpasses that of the IA565 wild type in terms of biomass (20). Further, a disruption of the remaining *mrkB* gene, to give strain IA Δ T3, results in the inability to form a functional type 3 fimbria, resulting in an inability to form a biofilm (20).

We chose to look at the competitive indexes of these mutant strains compared to that of the wild type to see what effect these mutations would have on the ability of IA565 to form a stable biofilm with PAO1. A competitive index (CI) of greater than 1.0 would indicate that the *K. pneumoniae* strain is found in greater numbers in the dual-species biofilm than PAO1, while a CI of less than 1.0 would indicate greater numbers of PAO1 cells. The CI of a biofilm composed of PAO1 and IA565 is around 0.25 (Fig. 1), indicating that

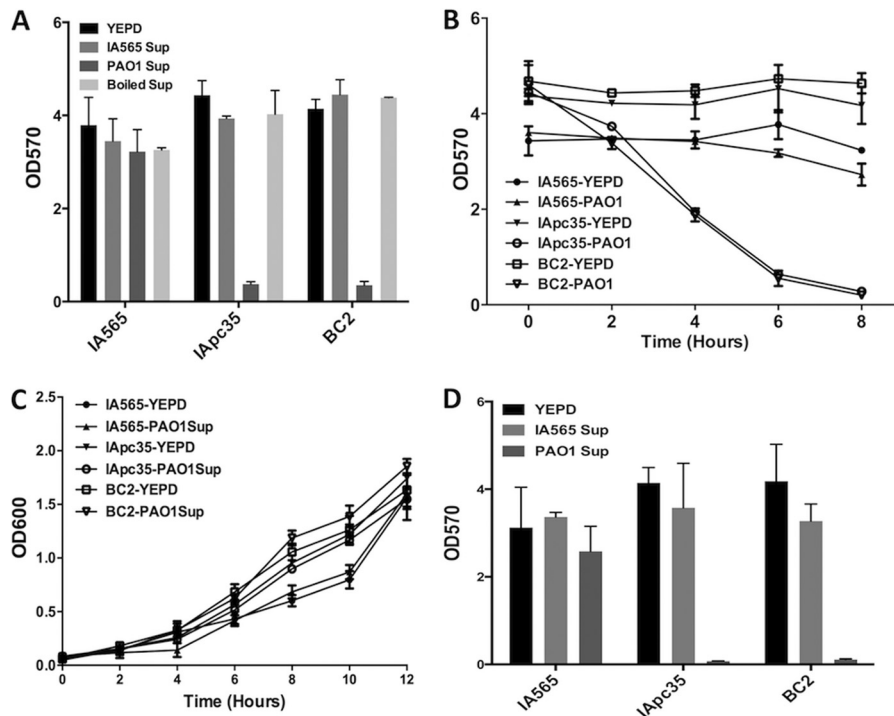


FIG 2 A heat-sensitive effector from *P. aeruginosa* PAO1 supernatant disrupts established biofilms from *K. pneumoniae* strains IApc35 and BC2. (A) IA565, IApc35, and BC2 biofilms, preformed in YEPD in 96-well microtiter plates at 37°C with shaking, were treated by replacing the medium with fresh reconstituted YEPD or IA565 or PAO1 supernatant or with reconstituted PAO1 supernatant boiled for 15 min. Biofilms were stained with crystal violet and resuspended, and the OD₅₇₀ was measured. (B) Dispersal of preexisting IApc35 and BC2 biofilms by *P. aeruginosa* PAO1 supernatant is completed in 8 h. Sixteen-hour biofilms of *K. pneumoniae* IA565, IApc35, and BC2 were treated with either YEPD or *P. aeruginosa* PAO1 supernatant in 96-well microtiter plates at 37°C with shaking. Biofilms were washed with crystal violet, and resuspended, and OD₅₇₀ readings were taken every 2 h. (C) Growth of planktonic *K. pneumoniae* was measured in the presence of either YEPD or PAO1 supernatant in 96-well microtiter plates at 37°C with shaking. OD₆₀₀ readings were taken every 2 h. (D) After growth of the *K. pneumoniae* strains was assayed, the resultant biofilms were assayed using crystal violet staining.

PAO1 makes up a vast majority of the biofilm biomass compared to IA565. As expected, a CI for the biofilm-deficient mutant IAΔT3 could not be generated, as no colonies were obtained (data not shown). Interestingly, the CI of mutant IApc35, which makes a stronger monospecies biofilm than IA565, generated a CI of 0.014, suggesting a more-than-10-fold decrease in competition compared to IA565. As IApc35 is a strain composed of numerous mutations, we sought to clarify the origin of sensitivity of IApc35 to PAO1. We created a transposon library of strain IA565 and identified mutants that were also defective for biofilm formation with PAO1. The mutant identified, BC2, carried an interruption in the *mrkD*_{1P} gene and exhibited a CI of 0.02 (Fig. 1), suggesting a role for the plasmid-borne fimbrial adhesin in forming a stable biofilm with *P. aeruginosa*.

Supernatant from *P. aeruginosa* PAO1 impairs biofilm development and disperses preexisting biofilm of *K. pneumoniae* strains lacking *mrkD*_{1P}. To determine if the antagonistic effect of PAO1 on IApc35 and BC2 populations in dual-species biofilms was the result of a secreted effector or a consequence of cell-cell contact, we tested the ability of the *K. pneumoniae* strains to form and maintain a biofilm in the presence of PAO1 supernatant. IA565, IApc35, and BC2 were allowed to form biofilms in a 96-well microtiter plate and were then treated with lyophilized and reconstituted versions of YEPD, IA565 supernatant, or PAO1 supernatant for 16 h with shaking at 37°C. While YEPD and IA565 supernatant had no effect on the stability of any of the preformed biofilms, PAO1 supernatant

dramatically decreased the biofilms of IApc35 and BC2, causing a >100-fold decrease in biomass (Fig. 2A). PAO1 supernatant had no effect on the stability of preformed IA565 biofilm (Fig. 2A). This activity was abolished by boiling the PAO1 supernatant for 15 min (Fig. 2A), suggesting that a heat-sensitive secreted effector from PAO1 played a significant role in the ability of *P. aeruginosa* to outcompete the *mrkD*_{1P}-deficient mutants of IA565. The activity of the PAO1 supernatant on the IApc35 and BC2 biofilms was fast acting, reaching maximum biofilm detachment by 8 h posttreatment (Fig. 2B). The ability of IA565, IApc35, and BC2 to form biofilm in PAO1 spent medium was tested by growing the strains in the same reconstituted YEPD or PAO1 supernatant. While IA565 was able to form biofilm in both types of media, IApc35 and BC2 were not (Fig. 2D), demonstrating that the supernatant disrupted pre-established biofilms and inhibited the formation as well. In order to test if the secreted effector was bactericidal in nature, we tested the growth kinetics of IA565, IApc35, and BC2 in reconstituted YEPD or PAO1 supernatant. The supernatant of PAO1 had no significant effect on the planktonic cell growth of any of the *K. pneumoniae* strains (Fig. 2C), suggesting that the PAO1 secreted effector was abolishing the biofilms through detachment and not killing.

The *mrkD*_{1P} gene was also used to complement strains IApc35 and BC2, and biofilms composed of these complemented strains were tested for biofilm disruption by PAO1 supernatant. IApc35

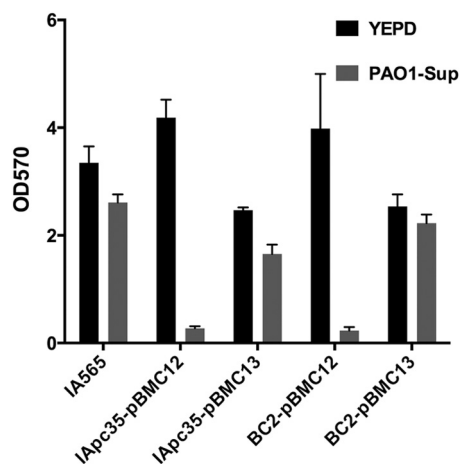


FIG 3 Complementation of IApc35 and BC2 with the plasmid-borne *mrkD*_{1P} restores biofilm resistance to PAO1 supernatant. Preformed biofilms of wild-type IA565 or mutant forms carrying either an empty vector (pBMC12) or *mrkD*_{1P} complement (pBMC13) were treated with YEPD or PAO1 supernatant for 16 h, and remaining biofilm was assayed by crystal violet stain and OD₅₇₀ reading.

and BC2 carrying an empty vector (pBMC12) displayed the same biofilm phenotype that is sensitive to supernatant from PAO1 (Fig. 3). Those same strains carrying a plasmid with *mrkD*_{1P} (pBMC13) shared the same phenotype of resistance to biofilm disruption as the wild-type IA565 (Fig. 3), suggesting that MrkD_{1P}, and not MrkD_{1C}, provides IA565 with a phenotype of resistance to biofilm disruption mediated by PAO1.

The secreted elastase B (LasB) from PAO1 disrupts preformed biofilm of *K. pneumoniae* IA565 lacking *mrkD*_{1P}. To identify the effector(s) responsible for the biofilm-disrupting ability of PAO1, the PAO1 two-allele library from the Washington Genome Center (37) was screened for mutants whose supernatants were unable to disrupt IApc35 preestablished biofilms. Strains from the library were grown in a fashion similar to that for wild-type PAO1, and the collected supernatant was used directly to assay biofilm dispersion. We identified nine mutants that were defective for the ability to disrupt IApc35 and BC2 preestablished biofilms (Fig. 4). While mutants from this library may harbor polar mutations, great care has been taken to reduce this possibility by the addition of a neomycin phosphotransferase promoter to the downstream end of the transposon (37). The disrupted genes of the identified mutants represent proteins that either have unknown function (PA0697) or have functions with unknown relationships to virulence in PAO1 (PA0502/BioH, PA1684/MtnD, and PA3383). Others identified are likely related to the transcriptional regulation of the secreted effector(s) involved in biofilm disruption (PA3135, PA0401, and PA4280). PA2478, a thiol: disulfide interchange protein, is a class of protein that is necessary for the proper folding and functionality of secreted proteins. The only secreted protein identified in the library screen was elastase B (PA3724/LasB), a zinc metalloprotease involved in host tissue damage, invasion, and immunomodulation, which has also recently been shown to play a role in the dispersal of *Staphylococcus aureus* biofilms. To test the effect of LasB protease on *K. pneumoniae* biofilms, purified LasB was mixed into YEPD medium and added to preformed IA565, IApc35, and BC2 biofilms. LasB had an effect on the *mrkD*_{1P}-deficient strains of *K. pneumoniae* iden-

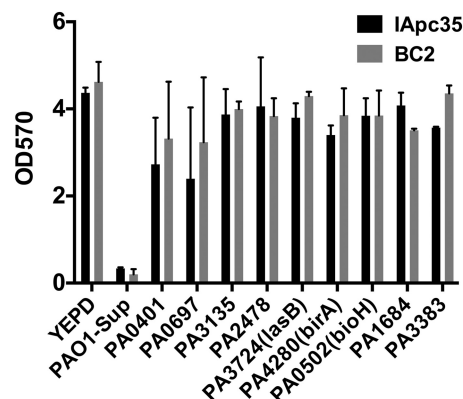


FIG 4 Biofilm dispersal activity of PAO1 transposon mutants. IApc35 and BC2 biofilms, preformed in YEPD in 96-well microtiter plates at 37°C with shaking, were treated after 16 h by replacing the medium with fresh YEPD or supernatant from PAO1 or transposon mutants. Biofilm formation was measured by staining with crystal violet and OD₅₇₀ measurement.

tical to that of PAO1 spent medium and also had no effect on wild-type IA565 (Fig. 5A). The results indicate that the protease LasB plays a role as an antibiofilm effector and that MrkD_{1P} likely plays a protective role against that activity.

Effects of proteases and other antibiofilm agents on *K. pneumoniae* biofilms lacking *mrkD*_{1P}. Proteases, including LasB, have previously been shown to have biofilm-dispersant activity with various microorganism (15). To gain a better understanding of the role of LasB in the disruption of *K. pneumoniae*, we also tested various other proteases, including elastases of different sources, trypsin, chymotrypsin, and proteinase K. Each of these proteases had activity nearly identical to that of LasB, eradicating preformed IApc35 and BC2 biofilms while leaving the wild-type IA565 biofilm intact (Fig. 5A). Boiling of these agents destroyed the antibiofilm activity (data not shown). A mixture of antichymotrypsin and chymotrypsin in YEPD failed to disperse IApc35 and BC2 biofilms, indicating that protease activity is necessary for biofilm disruption (see Fig. S1A in the supplemental material). When IApc35 and BC2 were complemented with a *mrkD*_{1P}-carrying plasmid, the resultant biofilms were protected from chymotrypsin-mediated attack (see Fig. S1B in the supplemental material). Most interesting is that *mrkD*_{1P}-deficient strains of IA565 are sensitive to elastases (e.g., elastases from polymorphonuclear leukocytes) and proteases produced by the colonized host (Fig. 5A), suggesting that these mutants may be unable to properly colonize a host or cause a chronic wound infection. Further study will clarify this phenotype.

In order to fully analyze the sensitivity of mutant *K. pneumoniae* biofilms, we also assayed the ability of these *mrkD*_{1P} mutants to maintain a biofilm in the presence of various other antibiofilm agents and antibiotics compared to that of the wild-type strain. The enzyme DNase and the fatty acid *cis*-2-decenoic acid, both of which have been shown to disrupt or detach biofilms (30, 38), had no effect on any of the preformed biofilms (Fig. 5B). β -Lactamase resistance in *K. pneumoniae* is a growing problem in the medical community (18). IA565 is highly resistant to β -lactamases such as carbenicillin, and this resistance is amplified when the organism is in a biofilm community (data not shown). Carbenicillin levels as high as 3 mg ml⁻¹ had no effect on any of the preformed biofilms (Fig. 5B), suggesting that biofilm-mediated

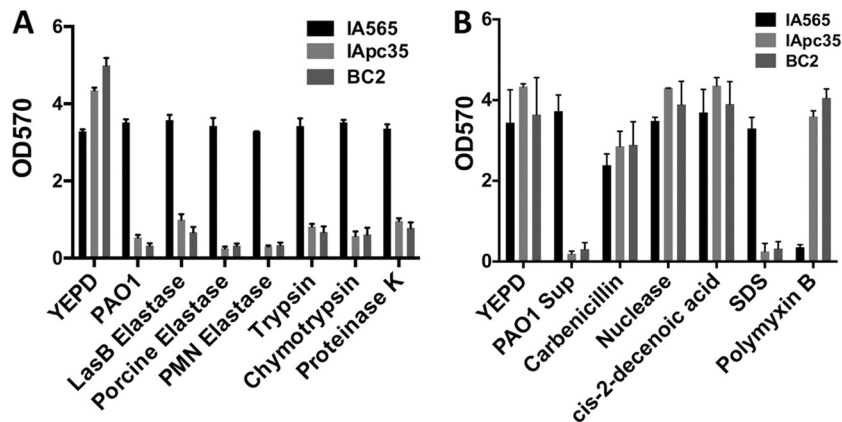


FIG 5 Biofilm dispersal activity of various proteases and antibiofilm agents. IApc35 and BC2 biofilms, preformed in YEPD in 96-well microtiter plates at 37°C with shaking, were treated after 16 h by replacing the medium with fresh YEPD or YEPD supplemented with the compounds listed. Biofilm formation was measured after 16 h by staining with crystal violet and OD₅₇₀ measurement.

antibiotic resistance is unaffected by the loss of MrkD_{1P}. However, sensitivity to SDS, a detergent, showed a pattern similar to that of LasB and other proteases (Fig. 5B). Interestingly, IApc35 and BC2 biofilms showed an increased resistance to the detergent-like polymyxin B, an antimicrobial that targets and disrupts the bacterial cell wall (Fig. 5B). The sensitivities and resistances demonstrated by the mutant strains of *K. pneumoniae* compared to wild-type IA565 suggest a change in the morphology and structure of the individual cell, the biofilm community, or both. To investigate these possibilities, we looked at cell morphologies of IA565, IApc35, and BC2 cells, grown under biofilm-inducing conditions, using scanning electron microscopy. The IA565 cell morphology was found to be highly elongated, creating cells of around 12 μm (Fig. 6). This morphology, however, was dramatically decreased in the mutant BC2 strain, creating cell lengths of less than 2 μm on average. Cell length was restored by complementation with the *mrkD*_{1P}-carrying plasmid pBMC13 (Fig. 6), suggesting that *mrkD*_{1P} may play a role in the development of an elongated cell morphology (representative micrographs are shown in Fig. S2 in the supplemental material). These changes in cell morphology likely represent a pleiotropic effect from the deletion of *mrkD*_{1P} that leads to the sensitivity/resistance pattern seen in *mrkD*_{1P}-deficient mutants, and elucidation of these mechanisms is still required.

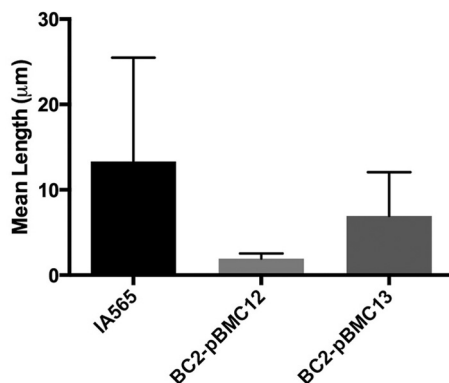


FIG 6 Mean cell length of *K. pneumoniae* wild-type strain IA565 and *mrkD*_{1P}-deficient strain BC2 with and without *mrkD*_{1P} complementation.

Role of *mrkD* in protection against protease-mediated biofilm disruption. Biofilm morphology, cell morphology, antibiotic resistance, and various other physiological features of *K. pneumoniae* can exhibit large degrees of variation between two strains (39). In order to examine the roles of MrkD_{1C} and MrkD_{1P} in providing biofilm-mediated resistance to protease-mediated dispersion, we looked at a number of other *K. pneumoniae* strains, carrying either MrkD_{1C} or MrkD_{1P} or both (Table 1), in the presence of porcine pancreas-derived elastase. As expected, UI917 and UI925, both carrying *mrkD*_{1P} but not *mrkD*_{1C}, were resistant to protease-mediated biofilm disruption (see Fig. S3 in the supplemental material). However, only two strains of *K. pneumoniae* carrying a *mrkD*_{1C} gene but no *mrkD*_{1P} gene showed any sensitivity to PAO1 supernatant (Fig. 7; see Fig. S3 in the supplemental material). The sensitive strains, THK12113 and CAS55 (C3091), showed a restored resistance to protease treatment with *mrkD*_{1P}-complementing plasmid pFK52 (Fig. 7). All strains were tested for planktonic growth and found to have growth kinetics similar to

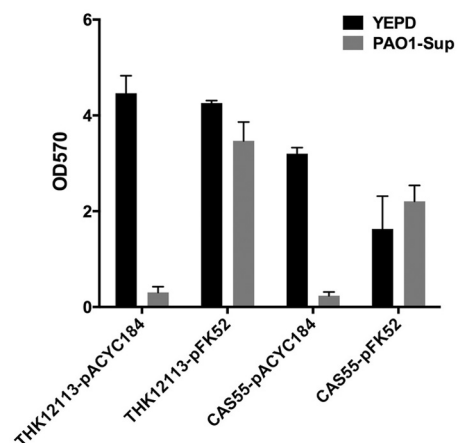


FIG 7 Complementation of CAS55 and THK12113 with the plasmid-borne *mrkD*_{1P} restores biofilm resistance to PAO1 supernatant. Preformed biofilms of CAS55 and THK12113, carrying either an empty vector (pACYC184) or *mrkD*_{1P} complement (pFK52), were treated with YEPD or PAO1 supernatant for 16 h, and remaining biofilm was assayed by crystal violet stain and OD₅₇₀ reading.

that of IA565 (see Fig. S4 in the supplemental material). These data suggest that in other strains, various factors besides *mrkD* may be involved in the protection of *K. pneumoniae* biofilms from protease-mediated attack.

DISCUSSION

Naturally occurring biofilms in the environment and the host are frequently found as multispecies communities that differ vastly in composition, structure, and antimicrobial resistance from monospecies biofilm systems (10). It is essential to look at these heterogeneous populations in order to gain a better understanding of the interactions that different microbial species have with one another in this complicated environment. Understanding these interactions will lead to identification of novel targets and therapeutics that likely would be missed otherwise. *K. pneumoniae* and *P. aeruginosa* are two organisms that are often found together in biofilm-mediated chronic wound infections (10), which are a growing burden on the medical community and cost millions of dollars every year in treatment (40). Understanding the interactions of these two species is essential in eliminating each as a common hospital-acquired infectious agent.

P. aeruginosa is an antagonistic organism that uses a number of effectors to gain a competitive advantage over other organisms, in both sessile and planktonic cultures. These effectors include LasA, LasB, *N*-homoserine lactone, pyocyanin, *cis*-2-decenoic acid, and rhamnolipids, which can kill or hinder the growth of competing bacteria or disrupt and disperse bacteria from heterogeneous biofilm communities (15). These effects have been demonstrated with various types of microorganisms, including *Candida albicans*, which is sensitive to phenazine (41), and *Staphylococcus aureus*, which is sensitive to LasB (15). However, it has been shown that *K. pneumoniae* is able to form a stable biofilm with *P. aeruginosa* (32), suggesting potential mechanisms used by *K. pneumoniae* to combat these effectors, which may also provide potential targets that could lead to the disruption of biofilms composed of this bacterium. The use of these potential therapeutics could lead to a drastic change in the population dynamics of the biofilm structure, which could allow for greater access of more common antimicrobial agents.

Many *K. pneumoniae* strains form biofilms using a type 3 fimbria that is produced and used by various members of the *Enterobacteriaceae* (42). These fimbriae are composed of subunits of the protein MrkA and accentuated at the tip by the fimbrial adhesion MrkD (19). Each of these, and other components, is chaperoned into place by the protein MrkB, and a knockout of the *mrkB* gene results in a strain lacking fimbriae and incapable of forming biofilm (20). The strain IA565 carries two copies of the *mrkD* gene (forms 1C and 1P) that are used for adherence to various forms of extracellular matrix proteins, such as collagen. A strain of IA565, IAp35, that lacks the plasmid-borne *mrkD*_{1P} gene and has a translation termination codon in the coding sequence of the chromosomal *mrkD*_{1C} actually forms a more robust biofilm than the wild type (20). However, this strain has an ~10-fold decrease in its ability to form a biofilm with the *P. aeruginosa* strain PAO1, and a monospecies biofilm composed of IAp35 is almost completely dispersed by the application of spent medium from PAO1. Using a transposon screen, we identified a *mrkD*_{1P} gene disruption, BC2, that produced an identical phenotype in relation to IAp35 in its reaction to the presence of PAO1 or PAO1 spent medium. Complementation of the *mrkD*_{1P} gene partially restored the resistance

of IAp35 and BC2 biofilms to that of the wild-type IA565, suggesting that *mrkD*_{1P}, but not *mrkD*_{1C}, is required for resistance to the effects of PAO1. This also suggests a potent target in *K. pneumoniae* that could be exploited in the future to help combat both single- and mixed-species infections. In fact, antibodies directed at MrkD were shown to protect against *K. pneumoniae* lung infection, providing a possible course of treatment using MrkD-specific antibodies in conjunction with other antimicrobials to help treat infection.

The ability of the PAO1 spent medium to disrupt preformed biofilms and inhibit their formation suggests that a secreted effector(s) is being used by PAO1 to attack *K. pneumoniae* biofilm. A screen of the PAO1 two-allele library from the Washington Genome Center identified elastase B (LasB) as the likely effector. Elastase B is a metalloprotease used by PAO1 for host invasion and biofilm dispersal in other microorganisms. The effect was not relegated only to LasB, as many other forms of proteases were able to emulate the disruptive effect. Most interestingly, leukocyte-I elastase was shown to have an effect on *K. pneumoniae* biofilms that was identical to that of LasB, suggesting that the human host may already be primed to fight *K. pneumoniae* infection. Targeting of MrkD_{1P} in *K. pneumoniae* biofilms could provide a key component in the host fight against infection.

The disruption of *mrkD*_{1P} also allowed for greater susceptibility to SDS, although it had no effect on the susceptibility to other biofilm-disrupting agents such as *cis*-2-decenoic acid or nucleases or to β -lactam antibiotics such as carbenicillin. Most interestingly, the biofilms of the mutant forms of IA565 showed increased resistance to polymyxin B, suggesting that a mechanism other than biofilm permeability may be at play in the altered resistance/susceptibility of IAp35 and BC2. Scanning electron microscopy revealed an altered cell morphology of the *mrkD*_{1P} mutants of IA565, showing an inhibition of cell elongation that occurs quite frequently in the wild type.

It is possible that the absence of MrkD_{1P} may cause a pleiotropic change in the cell surface content of IA565, either by changing cell surface dynamics or by altering the expression of cell surface-associated proteins or compounds. This may allow for decreased or increased access of antimicrobial agents, proteases, etc. These potential changes in cell surface content, extracellular matrix, and cell morphology caused by the deletion of *mrkD*_{1P} may inhibit the creation of a robustly interconnected community of bacterial cells that could allow for easier access of proteases that might otherwise be excluded. In these cases, it is likely that the MrkD protein is not directly mediating protection from proteolytic cleavage but rather is altering the biofilm dynamic in such a way as to create a protective environment for the bacteria. Further study is necessary to find the root cause of this phenotype.

While MrkD_{1P} appears to be required for competition with PAO1 and protection against protease-mediated biofilm disruption, this does not appear to be the case with various other forms of *K. pneumoniae*. While protected from the same protease treatments, the different forms of MrkD had no effect on the resistance phenotype. The cell morphologies and biofilm physiologies of these various strains can differ markedly, and it is likely that other factors are being used to provide the same type of resistance in various *K. pneumoniae* strains. Identification of these targets may also provide a similar route to the antibiofilm sensitivity found in the IA565 mutants IAp35 and BC2. It is important to understand these interactions of *K. pneumoniae* and *P. aeruginosa* further to

identify better ways of preventing and treating chronic wound infections.

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